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Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean

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Summary

We investigated whether termination of transcripts with a self-cleaving ribozyme can enhance nuclear retention and serve as a tool to decrease specific plant gene expression. Nuclear retention was first monitored in tobacco using the β-glucuronidase gene terminated with either the 35S CaMV 3′ untranslated sequence (UTR) or a *cis*-acting ribozyme. Northern blot analysis of nuclear RNA and total RNA, and *in situ* hybridizations showed that the ribozyme-terminated transcripts were preferentially retained in the nucleus of transgenic tobacco. Ribozyme-terminated transcripts were subsequently tested as a gene down-regulation strategy in soybean. The embryo-specific Δ-12 fatty acid desaturase *FAD2-1* gene was targeted because its down-regulation elevates oleic acid content of seed storage lipids. Both ribozyme-terminated antisense and standard antisense constructs were capable of gene down-regulation, producing over 57% oleic acid compared with less than 18% in wild-type seed. Ribozyme termination cassettes were also constructed to evaluate sense transcripts for single gene down-regulation and the simultaneous down-regulation of two embryo-specific genes in soybean using a single promoter. Eight independent soybean transformants were screened that harboured standard plus sense or ribozyme terminated *FAD2-1* cassette. Two of the eight ribozyme terminated transformants displayed oleic acids levels in the seed storage lipids of over 75%, while none of the standard plus sense *FAD2-1* lines showed elevated oleic acid phenotypes. The dual constructs targeted *FAD2-1* and the *FatB* gene encoding a palmitoyl-thioesterase. Five transgenic soybean lines harbouring the dual constructs had oleic acid levels, greater than 85%, and saturated fatty acids levels, less than 6%. Thus, ribozyme termination of transcripts can be utilized to specifically down-regulate endogenous gene expression in soybean.

Keywords: Gene silencing, nuclear retention, *Glycine max*, transformation.

Introduction

Attenuating the expression of endogenous plant genes is an important strategy for biotechnological modification of plant traits and ascertaining gene functionality. Antisense (Ecker and Davis, 1986), sense cocuppression (Matzke and Matzke, 1995) and RNA interference (RNAi) (Sharp and Zamore, 2000) have all shown some effectiveness at reducing gene expression in plants. Current models indicate for these down-regulation strategies that a post-transcriptional gene silencing (PTGS) mechanism is triggered in the cytoplasm (Marx, 2000; Matzke *et al.*, 2001; Waterhouse *et al.*, 1998; Wolffe and Matzke, 1999). However, in animal cells, nuclear retention of antisense transcripts is an effective strategy that increased the efficacy of viral gene down-regulation (Liu and Carmichael, 1994; Liu *et al.*, 1994). This was carried-out by replacing the 3′ untranslated region (UTR) with a self-cleaving ribozyme (RZ) derived from a satellite RNA of tobacco ringspot virus (Haseloff and Gerlach, 1988). The absence of an appropriate 3′ UTR prevented nuclear export of the transcript to the cytoplasm, and the RZ promoted generation of a free RNA from the precursor transcript.

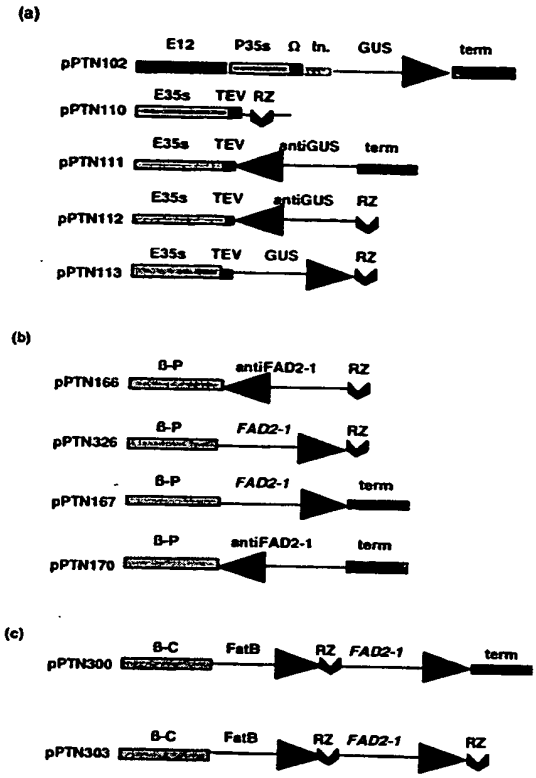

Figure 1. Gene of interest cassettes of the binary vectors.
(a) GUS cassettes in the respective binary vectors used for tobacco transformations pPTN102, pPTN110, pPTN111, pPTN112 and pPTN113.
(b) *FAD2-1* cassettes in the respective binary vectors of pPTN166, pPTN170, pPTN167 and pPTN326.
(c) Dual *FatB* and *FAD2-1* cassettes in the binary vectors pPTN300 and pPTN303. Abbreviations are: E35S-enhanced CaMV 35S promoter, TEV-tobacco etch translational enhancer, T35S-3′ CaMV UTR, P35S CaMV 35S promoter, RZ-ribozyme, *GUS-GUS* ORF, antiGUS-*GUS* ORF in antisense orientation, *FAD2-1*- *FAD2-1* gene, antiFAD2-1-*FAD2-1* gene in antisense orientation, β-P-Phaseolin promoter, β-C- β-conglycinin promoter, *FatB*-*FatB* gene in sense orientation.

To our knowledge the down-regulation of genes implementing RZ-terminated transcripts has not been reported in plants. The initial objective of this research was to determine if replacing standard 3′ UTR with a RZ would preferentially retain mRNA transcripts in the nucleus of plant cells. The subsequent objective of this work was to test whether RZ-terminated transcripts could specifically down-regulate plant gene expression. To this end we targeted soybean fatty acid gene expression, namely the embryo-specific, thioesterase *Fat*B gene (Kinney, 1997) and desaturase *FAD2-1* gene (Heppard *et al.*, 1996). Down-regulation of *Fat*B would result in decreased levels of saturated fatty acids, primarily a reduction in palmitate, whereas down-regulation of *FAD2-1* results in elevated levels of oleate and a reduction in polyunsaturated fatty acids. High oleate and/or low saturated fatty acid phenotypes were chosen because fatty acids are relatively easy to quantify and high oleate oil is a commercially desirable product with health benefits, increased economic value and enhanced industrial applications.

Results

Replacement of the 3′ UTR with a self-cleaving ribozyme results in GUS transcripts accumulating predominately in the nucleus

The *GUS* coding region was used as a convenient sense or antisense RNA, although *GUS* expression was not relevant for this localization experiment. Four constructs were assembled with the *GUS* coding region (ORF), along with a negative control lacking *GUS* (pPTN110) (Figure 1a). Plasmid pPTN102 (Figure 1a) contains a standard *GUS* expression cassette. Plasmid pPTN111 (Figure 1a) harbours an antisense *GUS* ORF coupled with the 3′ UTR of the 35S CaMV transcript, while vectors pPTN112 (Figure 1a) and pPTN113 (Figure 1a) carry the *GUS* ORF in antisense and sense orientation, respectively, coupled with the RZ at the 3′ end. Transgenic tobacco plants were produced containing these constructs to determine whether replacing the 3′ UTR with a self-cleaving RZ would retain *GUS* transcripts in the nucleus.

The subcellular location of the *GUS* transcripts in tobacco transformants was first determined by isolating either total or nuclear RNA from 15-day-old seedlings. Total RNA contains all cytoplasmic, nuclear and organellar RNA, while the RNA prepared from isolated nuclei has nuclear and organellar RNA. Figure 2(a) shows the staining of the nuclei that were isolated during the nuclear RNA preparation. The large red bodies are intact nuclei and the smaller yellow-green structures are chloroplasts. A northern blot analysis was subsequently performed using the TEV-GUS element as a probe (Figure 2b). All samples were standardized with respect to tissue type and age, and RNA loading. Based on the RNA recovered per fresh tissue, and assuming equal RNA recovery from nuclear and total RNA isolations, we estimate that approximately 5% of the cellular RNA was in the nuclear extract.

The pPTN102 and pPTN111 transformants, with RNAs containing a 3′ UTR that should confer cytoplasmic localization, produced more hybridization signals in the total RNA lanes than the nuclear RNA lanes in the northern analysis (Figure 2b). The nuclear RNA lanes produced stronger hybridization signals for the RZ-harbouring RNAs produced from transformants containing either the pPTN110, pPTN112, and pPTN113 constructs (Figure 2b).

These results suggest that the RZ-harbouring RNAs are predominately located in the nucleus.

The second approach to ascertain the subcellular location of the *GUS* transcripts was to use *in situ* hybridization. The results for the studies on transgenic individuals harbouring the two antisense constructs, pPTN111 or pPTN112, which contain either 3′ 35S CaMV UTR or RZ, respectively, are shown in Figure 3. The red channel shows nuclei staining with propidium iodide and the green channel shows the *in situ* hybridization of strand-specific probes. A comparison of the pPTN111 and pPTN112 antiGUS constructs shows hybridization by the sense probe in both the cytoplasm and nucleus in the

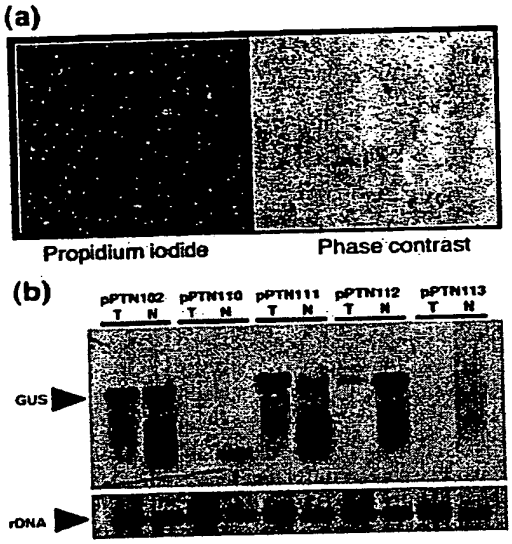

Figure 2. Northern analysis on tobacco transformants.
(a) Image analysis of nuclei-enriched fraction from tobacco. Left: propidium iodide image. Right: phase contrast of same region pictured on the left.
(b) Northern blot of transgenic tobacco harbouring pPTN102, pPTN110, pPTN111, pPTN112 and pPTN113. Total RNA lanes labelled T and nuclear RNA lanes are labelled N. The upper blot was probed with the TEV-*GUS* element and lower blot was probed with rDNA. The red asterisk indicates the TEV leader hybridization signal.

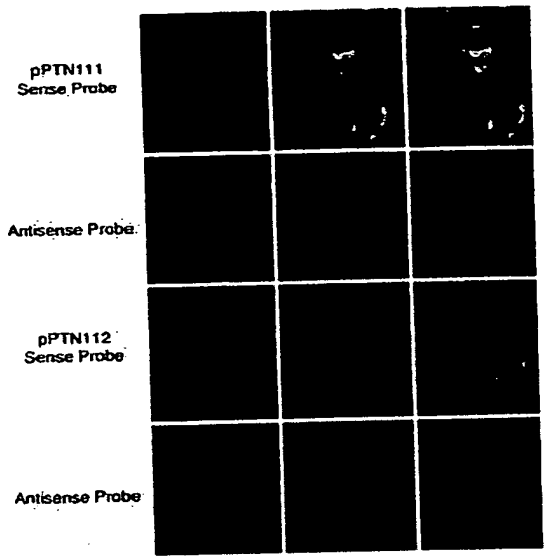

Figure 3. *In situ* hybridizations.
Left column represents the red channel image (nuclei). Center column represents the green channel (hybridization signal). Right column represents the combined images of red and green channels. Each row of images are arranged to show data from tobacco root tips harbouring pPTN111 or pPTN112 hybridized with either *GUS* sense probe or *GUS* antisense probe (control).

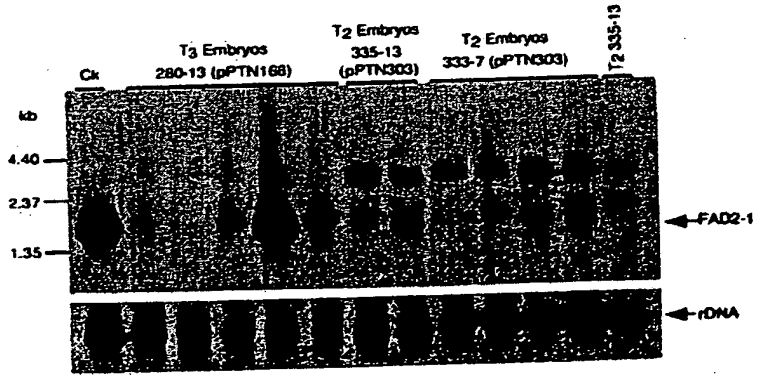

Figure 4. Northern blot analysis on immature soybean embryos.
Lane 1: wild type RNA. Lanes 2–6, RNA derived from immature embryos from transformant 280–13 (T_3 generation). Lanes 7, 8 and 13 RNA derived from immature embryos from event 335–13 (T_2 generation). Lanes 9–12 RNA derived from immature embryos from event 333–7 (T_2 generation).

pPTN111 tissue and a highly concentrated nuclear signal in the pPTN112 sample. This data is in agreement with northern analysis and demonstrates that the RZ-terminated transcripts are localized primarily in the nucleus.

A ribozyme-terminated transcript down-regulates the soybean FAD2-1 *gene*

The embryo-specific *FAD2-1* gene from soybean was chosen to determine if RZ-terminated transcripts, which are predominately localized in the nucleus, could be utilized as a strategy for endogenous gene down-regulation. A set of four binary constructs with either a 3′ UTR or a RZ, and containing either sense or antisense orientation of the *FAD2-1* coding region (Figure 1b) were transformed into soybeans. Soybean transformants at the T_0 stage were confirmed by Southern blot analysis and/or a herbicide tolerance phenotype (Data not shown). Seeds from the transformed plants were analysed for fatty acid composition by gas chromatography. A high oleic acid phenotype was observed in three of the four transgenic soybeans harbouring RZ-terminated antisense *FAD2-1* (pPTN166, Table 1). Soybean transformants 291–5, 280–13, and 288–9B had oleic acid levels of 62.3%, 57.7%, and 59.4%, respectively (Table 2). Wild type soybean oil had 17.9% oleic acid content.

A high oleic acid phenotype was observed in two of the nine transgenic soybeans harbouring the standard antisense *FAD2-1* construct (pPTN170) (Table 1). Soybean lines 294–14 and 294–5 yielded oleic acid levels of 61.7% and 82.1%, respectively (Table 2). No high oleic acid phenotype was observed across eight soybean transformants harbouring the 3′ UTR sense orientation *FAD2-1* element (pPTN167, Table 1). Two of the eight soybean transformants carrying the sense *FAD2-1*-RZ construct (pPTN326) displayed an elevated oleic acid phenotype (Table 1). Soybean transformants 374–1 and 374–4 possess oleic acid levels of 76.7% and 77.8%, respectively (Table 2). Therefore, in either sense or antisense orientation RZ terminated *FAD2-1* cassette under the control of

Table 1. Frequencies of high oleic acid phenotype in transgenic soybean lines. Vector column refers to the respective binary vector (see Materials and methods). Total No. Screened column indicates the total number of soybean lines assayed for elevated oleic acid phenotype. No. of HO lines column refers to the total number of soybean lines with elevated oleic acid phenotype. Percentage column indicates the percentage of lines screened with a high oleic acid phenotype

Vector	Total No. screened	No. of HO Lines	Percentage
pPTN166	4	3	75.0%
pPTN170	9	2	22.2%
pPTN167	8	0	0.0%
pPTN326	8	2	25.0%
pPTN300	15	6	40.0%
pPTN303	27	5	18.5%

Table 2. Seed fatty acid profiles in soybean transformants with high oleic acid phenotype. The ID column refers to the soybean transformant designation. WT indicates wild type soybean. Numbers within each of the fatty acid columns represents the mean percentage of the respective fatty acid. Wild type, null, progeny, from each of the respective transfromants with elevated oleic acid content were not included in the calculation of the means. Means within each column were separated by Fisher's LSD analysis. Means within each column followed by the same letter are not significantly different ($P = 0.05$). Transformants with palmitic acid underlined means indicate dual downregulated transformants

ID	Vector	% Palmitic	% Stearic	% Oleic	% Linoleic	% Linolenic
280–13	pPTN166	9.0c	3.9de	57.7b	21.2d	8.1d
288–9B	pPTN166	7.7b	3.8cde	59.4bc	19.2cd	8.5d
291–5	pPTN166	9.9c	3.0b	62.3bcd	15.9cd	8.6d
294–5	pPTN170	7.2b	4.2e	82.1fg	3.3a	3.1ab
294–14	pPTN170	9.0c	3.7cd	61.7bc	17.1cd	8.3ab
374–1	pPTN326	7.7b	3.7cd	76.7ef	5.1ab	6.5c
374–4	pPTN326	7.8b	3.4c	77.8ef	4.1a	6.6c
335–12	pPTN300	2.7a	3.0b	89.1gh	1.2a	2.9a
333–2	pPTN300	11.7e	3.6cd	68.4cde	8.3ab	7.7cd
333–3	pPTN300	11.7e	3.5cd	69.6de	8.0ab	7.0cd
333–51	pPTN300	10.1cd	4.1e	73.8e	5.2a	6.3c
333–8	pPTN300	11.5de	3.7cd	72.1e	5.6a	6.9c
325–61	pPTN303	2.8a	2.3a	89.1gh	1.4a	3.1ab
333–7	pPTN303	2.6a	2.9ab	89.4gh	1.5a	2.7ab
329–39	pPTN303	3.0a	2.6a	87.2gh	1.7a	4.5b
335–13	pPTN303	2.2a	2.5a	91.1 h	1.4a	2.1a
335–38	pPTN303	8.1b	3.8de	68.8de	12.3bc	6.9c
WT	–	13.1e	5.2f	17.9a	49.4e	14.4e

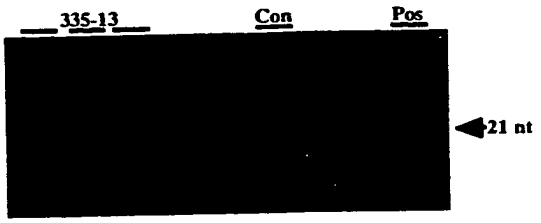

Figure 5. Detection of siRNAs in transformant 335–13.
Lanes 1 through 3: total RNA from immature T_3 seed of transformant 335–13. Lanes were loaded with approximately 18 μg total RNA. Con lane: 18 μg of total RNA from immature seed derived from a soybean transformant harbouring a 35S CaMV GUS cassette. Pos lane: 150 ng of a 21 nt oligio derived from *FAD2-1*, which served as a positive control and size marker.

the β-phaseolin promoter was able to effectively down-regulate endogenous *FAD2-1* expression.

Two genes can be down-regulated from a single promoter by nuclear localization

The soybean embryo-specific *Fat*B and *FAD2-1* genes were used to test the whether two genes can be simultaneously down-regulated from a single promoter, employing constructs designed for nuclear localization. Two binary constructs were assembled, each containing *Fat*B and *FAD2-1* in sense orientation and driven by a single embryo-specific promoter, β-conglycinin (Figure 1c). Both plasmids contain the RZ as a linker between the two genes, while pPTN300 is differentiated from pPTN303 in that the former is terminated by a normal 3′ UTR, and the latter is terminated by the RZ (Figure 1c).

All of the soybean transformants showing elevated oleic acid also displayed reductions in saturated fatty acids (Table 2). We assume this is due to the elevated oleate level out-competing palmitate in the acyltransferase reactions, thus reducing the saturated fatty acids on the glycerol backbone. Therefore, simultaneous down-regulation of *FatB* and *FAD2-1* was distinguished from *FAD2-1* independent down-regulation by comparing the mean percentage of palmitate, in the elevated oleic acid soybeans carrying the single gene vectors (pPTN166, pPTN170 and pPTN326), with the palmitate levels in the high oleate soybeans derived from the dual gene vectors (pPTN300 and pPTN303) (Table 2). A high oleic acid phenotype was observed in six of the 15 transgenic soybeans harbouring pPTN300 (Table 1). Of the elevated oleic acid transformants carrying pPTN300, only transformant 335–12 displayed a reduced saturate fatty acid phenotype suggesting dual down-regulation (Table 2). This transformant, 335–12, displayed oleic acid levels of 89.1% with the saturated fatty acids below 6% (Table 2). A high oleic acid phenotype was observed in five of the 27 soybean lines harbouring pPTN303 (Table 1). Four of the five lines possessed oleic acid levels of over 86%, and all of these showed significant reductions in the level of saturated fatty acids (Table 2), suggestive of dual gene silencing.

Northern blot analysis was conducted on a subset of the lines that displayed an elevated oleic acid phenotype in the seed storage lipids. The transformants analysed included five immature seed derived from a T_2 individual of transformant 280–13 (pPTN166), three immature seed derived from a T_1 individual of transformant 335–13 (pPTN303), and four immature seed from a T_1 individual of transformant 333–7 (pPTN303). An immature seed from wild-type soybean was included as a control. Four of the five samples from transformant 280–13 displayed significant reduction in *FAD2-1* transcript as compared to the control (Figure 4). We assumed that all five of the samples were not down-regulated because the parental plant was heterozygous. This assumption was subsequently confirmed by monitoring for segregation of herbicide tolerance in a larger population of T_3 individuals from the sampled T_2 individual (Data not shown). All the transformants assayed at the transcript level displayed a reduced *FAD2-1* message, which is in agreement with the fatty acid data derived from these transformants (Figure 4, Table 2).

The production of the characteristic small interfering 21–25 nt RNAs (siRNAs) of RNA silencing pathway (Vance and Vaucheret, 2001) were detected in total RNA preparations from three immature seeds from a T_2 individual of transformant 335-13 (Figure 5). An immature seed from a soybean transformant harbouring 35S CaMV *GUS* cassette served as a control in the experiment. The hallmark siRNAs were detected in all three RNA preparations, when the *FAD2-1* coding region was used as a probe, from 335-13 transformant suggesting that the mechanism of the down-regulation triggered by the RZ containing transcripts is the same for that described for RNA silencing that proceeds from transcripts terminated with a 3′ UTR.

Discussion

We have demonstrated that RZ-terminated transcripts are present predominantly in the nucleus of plant cells, in agreement to what has been observed in animal cells (Eckner *et al.*, 1991; Liu and Carmichael, 1994). However, our experiments don't address the possibilities that some of the RZ-terminated transcripts are present in the cytoplasm or that they are exported but unstable in the cytoplasm. We chose modification of soybean oil with elevated oleate and/or reduced saturates as target phenotypes to test whether RZ-terminated transcripts can specifically down-regulate gene expression in plants. Although the total number of independent transformants is small, in each case a higher percentage of elevated oleic

acid phenotype was observed in soybean transformants that harboured the RZ- terminated *FAD2-1* cassette (pPTN166 and pPTN326) than those carrying the 3′ 35S CaMV UTR *FAD2-1* cassette (pPTN170 and pPTN167) (Table 1). These data demonstrate that RZ-terminated transcripts can specifically down-regulate gene expression in plant cells.

Simultaneous down-regulation of two genes utilizing the RZ-mediated nuclear retention approach was successful. The binary vectors pPTN300 and pPTN303 differ only in the termination element of the *FAD2-1* ORF (Figure 1c). The data revealed elevated oleic acid phenotype in 40% of the pPTN300 transformants and 18.5% of the pPTN303 transformants (Table 1). However, only a subset of the high oleate soybeans harbouring pPTN300 or pPTN303 displayed down-regulation of *FatB* (Table 2). Coordinated down-regulation of two genes from a single promoter was reported in tomato (Jones *et al.*, 1998). In this study polygalacturonidase (*Pgu*) and phytoene synthase (*PSY*) cDNA fragments were assembled in two cassettes under the control 35S CaMV promoter coupled with the 3′ 35S CaMV UTR. The *Pgu* fragment resided 5′ in both cases, while the *PSY* element was oriented in either sense or antisense downstream of the *Pgu* fragment. In all cases in which down-regulation of *PSY* was observed, *Pgu* expression was co-ordinately suppressed (Jones *et al.*, 1998). In contrast, in soybean transformants harbouring the dual *FatB*/*FAD2-1* coupled with the RZ, we observed both co-ordinated down-regulation, and independent *FAD2-1* suppression. Reduction in *FatB* alone was not observed. This suggests that the RZ processed transcripts in the dual gene cassettes triggered down-regulation of the respective endogenous genes independently.

There are several lines of evidence that PTGS can occur in the nucleus. Wang and Waterhouse (2000) have postulated that a feature of a silencing RNA is its localization within the nuclear compartment. In a study designed to down-regulate a transgenic *GUS* gene in rice, they observed a build-up of unpolyadenylated defective *GUS* transcripts in the silenced transformants (Wang and Waterhouse, 2000). Mishra and Handa (1998) characterized tomato lines sense suppressed for pectin methyltransferase (PME) expression, and determined that the sense PME transgene inducing PTGS in tomato interfered with pre-mRNA processing in the nuclear compartment (Mishra and Handa, 1998). Bosher *et al.* (1999) targeted the introns of lineage abnormal genes, *lir-1* or *lir-26* of *C. elegans* via RNAi and concluded that the RNAi molecules can directly affect nuclear transcripts. Viroids are virus-like, self replicating RNAs, that do not encode for proteins and that replicate within the nuclear compartment via DNA-dependent RNA polymerase II (Schindler and Mülbach, 1992). The production of the hallmark siRNAs representing various domains of the potato spindle tuber viroid were observed in infected tomato plants (Papaefthimiou *et al.*, 2001). This suggests that an infectious RNA can induce a PTGS-like response that is initiated with in the nuclear compartment. We observed the accumulation of siRNAs representing homologous portions of the *FAD2-1* coding sequence in soybean transformant 335-13 (Figure 5). This suggests that RZ-terminated transcripts, which predominately accumulate in the nucleus, are mediating down-regulation mechanistically via the PTGS pathway (Vance and Vaucheret, 2001), indicating that nuclear retention of transcripts mediated by RZ processing can invoke PTGS in plant cells. However, an alternative explanation of these data is that the RZ-terminated transcripts serve as a preferred substrate for a cytoplasmically located RNA-directed RNA polymerase, which could rapidly generate dsRNA and be processed to siRNAs.

Models for PTGS in plants have emphasized the degradation of the target RNA species in the cytoplasm (Matzke *et al.*, 2001). This degradation is triggered by dsRNA molecules that are homologous to the target gene and ultimately generates the hallmark siRNAs that lead to RNA degradation. There may exist dual silencing apparatuses in plant cells, as has been previously hypothesized (Papaefthimiou *et al.*, 2001), one that resides in the cytoplasm and another in the nuclear compartment. If the transcript that generates the dsRNA, either through an aberrant RNA or antisense RNA intermediate, is exported to the cytoplasm, then the derived siRNA can mediate targeted RNA degradation in the cytoplasmically localized multicomponent silencing complex (Vance and Vaucheret, 2001). Conversely, if the transcripts are export incompetent, then the generated dsRNA and subsequent siRNA molecules are accumulated in the nuclear compartment which than serve as a guide for the sequence specific RNA degradation within the nucleus. If dual silencing complexes do exist in plant cells then this would provide an explanation for PTGS targeting of both pre-mRNA and mature mRNA for specific degradation.

Experimental procedures

Assembly of constructs

Plasmid cloning was performed using standard procedures (Sambrook *et al.*, 1989). The binary vector pPTN102 was constructed by ligating the *HindIII*/*EcoRI GUS* cassette from pE7131-*GUS* (Mitsuhara *et al.*, 1996) into pGPTV-hpt (Becker *et al.*, 1992). The 3′ 35S CaMV UTR in the plant expression vector pRTL2 (Carrington and Freed, 1990) was replaced by a self-cleaving RZ derived from the a satellite RNA of tobacco ringspot virus (Haseloff and Gerlach, 1988) from the plasmid pBS-RZ-2 (a gift from Gordon Carmichael) to yield pPTN106. The *GUS* open reading frame (ORF) was subcloned into pPTN106 to yield pPTN108 and pPTN109, which harboured RZ-terminated antisense and sense *GUS*, respectively. The vector pPTN107 is a derivative of pRTL2 containing the antisense *GUS* cassette with

the 3′ 35S CaMV UTR. The three *GUS* cassettes from pPTN107, pPTN108, and pPTN109, as well as the promoter-RZ element from pPTN106 were subcloned as *Hind*III fragments in to the binary vector pPZP112 (Hajdukiewicz *et al.*, 1994) to produce pPTN111, pPTN112, pPTN113 and pPTN110, respectively (Figure 1a).

The soybean *FAD2-1* ORF was isolated via PCR from genomic DNA (cv. A3237). PCR primers were designed from published sequence data (Heppard *et al.*, 1996). The primers were Fad2–5 (5′-TTTTTTCTAGAACTAGGCATGGGTCTAGC-3′) and Fad2–3 (5′-TTTTTGGATCCCCATCAATACTTGTTC-3′) with a *Xba*I site on 5′ primer and a *Bam*HI site on the 3′ primer. PCR reactions included 200 ng of total genomic soybean DNA as template, 1 × PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10 ng of each primer and 1 μl of Taq polymerase (Gibco Life Technologies, Rockville, MD, USA, Cat. #18038–042). PCR conditions were: 1 cycle for 5 min at 94°C; 2 cycles for 1 min at 94°C, 1 min 45°C, 2 min at 72°C; 35 cycles for 1 min 94°C, 1 min 55°C, 2 min at 72°C; and a 4°C holding temperature. The PCR product was digested with *Bam*HI and *Xba*I and subcloned into pBluescript KS + to yield pPTN156 and then sequenced for verification. Four expression cassettes were assembled with the *FAD2-1* ORF. All the cassettes were under the control of the common bean (*Phaseolus vulgaris* L.) embryo-specific β-phaseolin promoter (Frisch *et al.*, 1995). *FAD2-1* was subcloned downstream of the promoter in either sense or antisense orientation coupled with either 3′ 35S CaMV UTR or 3′ RZ. The respective cassettes were cloned into the binary vector pPTN163, a derivative of pPZP202 (Hajdukiewicz *et al.*, 1994), which carries a *bar* (Thompson *et al.*, 1987) cassette under the control of the 35S CaMV promoter. The resultant vectors, pPTN166, pPTN167, pPTN170, and pPTN326 harbour the *FAD2-1* cassettes antisense-RZ, sense-T35S, antisense-T35S, and sense-RZ, respectively (Figure 1b).

The soybean embryo-specific β-conglycinin promoter and *FatB* sense ORF were PCR amplified from pBS56 (provided by DuPont, Wilmington, DE, USA) using a 5′ primer TB-13 (5′-ATTACGAGCTCAAGCTTGATCCA-TGCCCTTC-3′), which contains *Sst*I and *Hind*III sites and is complementary to the upstream promoter element, and a 3′ primer TB-14 (5′-AATCGGAAT-TCAAATCTTAGGTGCTTC-3′), which contains an *Eco*RI site and is complementary to the 3′-end of the *FatB* ORF including the stop codon. PCR reactions included 100 ng of pBS56 with a reaction mix similar to that described above for *FAD2-1*. The PCR parameters were 2 cycles at 94°C for 1 min, 45°C for 30 sec, 72°C for 2 min followed by 7 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 2.5 min and then 1 cycle at 72°C for 5 min. The PCR product was digested with *Sst*I/*Eco*RI and fused to the RZ in the vector pPTN304, a derivative of pBS-RZ-2, to yield pPTN309.

The binary vector pPTN300 (Figure 1c) was constructed by engineering convenient restriction sites at the 5′ end of the *FAD2-1* ORF and the 3′ end of the 35S CaMV terminator via PCR utilizing pPTN167 as template DNA employing Pfu polymerase (Stratagene, La Jolla, CA, USA, Cat #600153). The PCR product from this reaction was subcloned into pPTN309 to generate the plasmid pPTN312, which harboured the cassette with *FatB*-sense/RZ/*FAD2-1* sense/T35S under the control of the β-conglycinin promoter. The β-conglycinin promoter from pPTN312 was subsequently subcloned to the binary plasmid pPTN130 that carries a 35S CaMV *bar* cassette to derive the plasmid pPTN300 (Figure 1c).

A final binary vector pPTN303 (Figure 1c) required a number of intermediate plasmids. Initially the engineering of convenient restriction sites at the 5′ and 3′ ends of the *FAD2-1* ORF was performed via PCR and the product subcloned to pBS-RZ-2 to yield pPTN305. The plasmid pPTN305 has the *FAD2-1* ORF with the RZ at the 3′ end. Additional restriction sites were added to the 5′ and 3′ ends of the FAD2-1-RZ element in pPTN305 via PCR and this product was fused down stream of the β-conglycinin-*FatB*-RZ element in pPTN309 to generate pPTN313. The β-conglycinin-*FatB*-RZ-*FAD2-1*-RZ cassette from pPTN313 was cloned into the binary vector pPTN130 to derive pPTN303 (Figure 1c).

Tobacco and soybean transformations

The binary vectors used in tobacco transformations (Figure 1a) were mobilized into *Agrobacterium tumefaciens* strain C58C1 carrying the Ti plasmid pMP90 (Koncz and Schell, 1986) by triparental mating (Ditta *et al.*, 1980). The *Agrobacterium* transconjugant carrying pPTN102 was selected on a LB agar plate supplemented 50 mg l^{-1} rifampicin, 50 mg l^{-1} gentamicin and 50 mg l^{-1} kanamycin. *Agrobacterium* transconjugants carrying pPTN110, pPTN111, pPPTN112, and pPTN113 were selected on LB agar plates supplemented with 50 mg l^{-1} rifampicin, 50 mg l^{-1} gentamicin, and 75 mg l^{-1} chloramphenicol.

The binary vectors used in soybean transformations (Figure 1b,c) were mobilized into *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986). The transconjugants carrying pPTN166, pPTN167, pPTN170, pPTN300, pPTN303 and pPTN326 were selected on LB plates supplemented with 50 mg l^{-1} kanamycin, 25 mg l^{-1} chloramphenicol, 100 mg l^{-1} streptomycin and 100 mg l^{-1} spectinomycin. The binary vectors in *Agrobacterium* were confirmed by plasmid preparations and restriction digestion.

Tobacco (*Nicotiana tabacum* genotype Xanthii) transformations were conducted via the leaf disc method described by Horsch *et al.* (1985). Transformants were selected on 10 mg l^{-1} hygromycin (pPTN102) or 150 mg l^{-1} kanamycin (pPTN110, pPTN111, pPTN112 or pPTN113). Soybean (*Glycine max* genotypes A3237 or Thorne) transformations were conducted using the cotyledonary-node transformation system (Hinchee *et al.*, 1988) using modifications previously described (Clemente *et al.*, 2000; Xing *et al.*, 2000; Zhang *et al.*, 1999). Transformants were selected on 5 mg l^{-1} glufosinate during shoot initiation and 3 mg l^{-1} glufosinate during shoot elongation steps.

Northern blot analyses

Homozygous tobacco lines carry the respective T-DNA elements described above were sterilized and plated on a modified MS medium (Murashige and Skoog, 1962) supplemented with either 15 mg l^{-1} hygromycin (pPTN102 events) or 100 mg l^{-1} kanamycin (pPTN110, pPTN111, pPTN112, and pPTN113 events). RNA isolations were conducted on 15-day-old seedlings. For total and nuclear RNA preparations the seedlings were ground in chilled extraction buffer (10 mM HEPES, pH 8.0, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) at a ratio of 300 mg tissue per ml buffer. The homogenate was mixed with 750 μl of Trizol LS (BRL) and the remaining extraction steps followed the manufacturer's protocol. For nuclear RNA preparations the homogenate was filtered through 33 μM nylon mesh and the filtrate subsequently centrifuged at 1310 *g* for 45 sec. The pellet was suspended in cold extraction buffer and re-centrifuged. The resulting pellet was suspended in 250 μl of cold extraction buffer. The preparation was then mixed with 1.1 ml of Trizol LS and the subsequent steps followed the manufacturer's protocol.

Total RNA was isolated from developing soybean embryos at approximately 30 days after flowering. Two hundred-300 mg of

tissue was used for total RNA extraction following the protocol outlined above.

Northern blot analysis was conducted on 10 µg of RNA. Samples were separated at 1.5 V cm^{-1} for 6 h on 1% agarose gels in 1 × MESA buffer, 0.3 M formaldehyde. The gels were washed twice in ddH_20 and twice in 2 × SSC. RNA was transferred to Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA, USA, Cat #162-0196) in 20 × SSC for 12 h and fixed via UV irradiation.

Filters were hybridized in 25 ml of 7% SDS, 0.5 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% BSA amended with 10–15 ng of denatured probe. Probes included *FAD2-1* ORF, TEV leader *GUS* ORF or a 400-bp fragment of the soybean 28S rDNA. Probes were labelled with [α-^{32}P] dCTP by random prime synthesis using Stratagene's Prime-It II kit (Cat # 300385) was conducted following the manufacturer's protocol. Hybridizations were conducted at 65°C.

Detection of siRNAs

Small interfering RNAs were detected following a modification of the protocol described by Hutvágner *et al.* (2000). Total RNA was isolated from immature seeds of a T_2 transformant derived from transgenic 335–13 as described above. The RNA (app. 18 µg. Note: Viscosity of the preps resulted in unequal loading of the wells) was separated on 15% TBE-urea gel (Bio-Rad cat #161–1117) for 3 h at 50 V. Following separation the RNA was electroblotted to Zeta-probe nylon membrane and fixed via UV irradiation. The membrane was hybridized, as described above, with the *FAD2-1* ORF at 40°C.

In situ *hybridization and confocal microscopy*

Tobacco root tips, taken from aseptic grown 2-week-old seedlings, were fixed in 4% paraformaldehyde in PBS for 2 h, followed by two10 min washes in PBS, two 10 min washes in PBS supplemented with 1 mg ml^{-1} sodium borohydride, and a final wash in PBS/Triton X-100 for 15 min. Following the washing steps the tissue as incubated overnight in PBS supplemented with sucrose at a final concentration of 30% at 4°C. The tissue was frozen and 8 µm sections collected on poly L-lysine coated slides, air dried and stored at −20°C until use.

The slides containing the sectioned tissue were treated with 0.2 N HCL for 5 min, proteinase-K for 10 min at 37°C, and acetic anhydride with 0.1 M triethanolamine for 10 min prior to pre-hybridization. The samples were incubated in pre-hybridization buffer (5 × SSC, 1 × Denhardt's solution, 1% SDS, 50 µg/ml denatured salmon sperm DNA, 50% formamide, 20 mM sodium phosphate pH 6.6) for 2 h at 50°C. Hybridizations with the *GUS* probes were conducted overnight at 50°C in the same buffer supplemented with 10% dextran sulfate. Sense and antisense-specific probes were DIG-labelled RNA that were polymerized and then hydrolyzed to <500 bases using the DIG labelling system.

Following the hybridization the samples were washed in 2 × SSC at 60°C for 10 min, followed by an RNAase A treatment for 10 min at 37°C, followed by a series of washes in 1 × SSC with one final wash in 0.2 × SSC at 37°C for 30 min. The samples were subsequently rinsed in TTBS (0.05% Tween 20, 1 × TBS (20 mM Tris, 0.5 M NaCl, pH 7.5)) and incubated with TTBS plus 5% BSA for 1 h. Samples were transferred to TTBS plus 1% BSA and FITC-conjugated sheep anti-DIG antibodies (1 : 5 dilution, Roche Molecular Biochemicals, Inc., Mannheim, Germany) for 2 h. Following the 2-h incubation the slides were washed twice in TTBS and once in TBS for 10 min each wash. The slides were subsequently stained with propidium iodide for 5 min, rinsed in TBS, and mounted immediately for image analysis. Images were collected with a Bio-Rad MRC1024ES confocal laser scanning microscope using a dual excitation (488/568 nm) and dual emission (520/598 nm) programme.

Fatty acid analysis

Seed chips from 4-8 individual seeds from either T_1, T_2, or T_3 generation of transgenic soybeans were used for fatty acid analysis. Fatty acid analysis was performed using gas chromatography according to the procedure of Butte *et al.* (1982). The remaining portion of the seed, with embryonic axis, was planted and plants allowed to grow to maturity in the greenhouse.

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